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13. ABSTRACT *(Maximum 200 Words)*

The breast tumor kinase BRK is not expressed in normal breast tissue, but is expressed in a high proportion of breast tumors and breast tumor cell lines. Because of its high expression in breast tumor cells, we propose that BRK regulates signaling that leads to protein expression that contributes to breast cancer development. We are currently examining changes in protein expression due to ectopic expression of BRK in the normal murine mammary gland cell line NMuMG. Availability of the human genome sequence has led to our discovery of tight linkage of the Srm tyrosine kinase gene to the Brk gene on human chromosome 20q13.3. Because of the tight linkage of these two genes, it is possible that they are coregulated in breast cancer cells. Expression of Srm in normal breast tissue and breast tumor cells is currently under investigation. While BRK has a low degree of homology to other intracellular tyrosine kinases including Srm, Frk/Rak, and Src42A/Dsrc41, the exon structures of these kinases is highly conserved, and distinct from other families of intracellular kinases including c-Src. Frk/Rak, is also expressed in breast cancers, suggesting a general role for the BRK family of kinases this disease.

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Introduction

The Intracellular Breast Tumor Kinase BRK

The human tyrosine kinase BRK (**Br**east tumor **k**inase) is an intracellular kinase of the form SH3-SH2-YK, where SH3 is a polyproline binding motif, SH2 recognizes phosphorylated tyrosine in a sequence specific context, and YK is the tyrosine kinase catalytic domain. Unlike members of the SRC family, BRK has a very short unique amino terminus and appears to lack a consensus myristoylation motif. While BRK is most closely related to members of the Src family, it is highly diverged, with nearly equivalent homology to the proto-oncogenes p60-YRK [Q02977]; p59-FYN [P27446]; p90 v-YES [61504] and its cellular homologue c-YES; FRK/RAK [P42685]; and c-Src itself, all with 44-45% protein identity. The BRK gene has only two intron boundaries conserved with the SRC family members (1, 2) suggesting that BRK is part of a distinct family of nonreceptor tyrosine kinases.

A fragment of the Brk cDNA was initially cloned from cultured normal human melanocytes using reverse transcription PCR with degenerate primers corresponding to the conserved regions of tyrosine kinase catalytic domains and named PTK6 (protein tyrosine kinase 6) (ACCESSION NM_005975) (3). A portion of the catalytic domain coding sequence was subsequently cloned using reverse transcription PCR and RNA isolated from involved axillary nodes from a patient with metastatic breast cancer (4). The full length BRK cDNA was then isolated from the MCF-7 and T-47D breast cancer cell lines (4). BRK cDNA clones were also isolated from a normal human small intestinal cDNA library (5).

BRK Expression in Breast Cancer

BRK has been detected in breast and colon tumors and in breast, colon and metastatic melanoma tumor cell lines (4-7). The sequence of BRK isolated from tumor cells and normal cells appears identical (1, 2), suggesting that BRK which is overexpressed in tumor cells is the normal protein.

BRK protein was present at high levels in T-47D, ZR75-30, BT-474, BT-20, MDA-MB-453, and MDA-MB-361 breast tumor cell lines; moderate levels in SKBR-3, MDA-MB-231 breast tumor cell lines and the MCF-10A breast epithelial line from a patient with fibrocystic disease, and at low or zero levels in the PMC42, MDA-MB-157, MDA-MB-468, and Cal51 breast tumor cell lines (6). While BRK expression could not be detected in normal breast tissue (5), it is expressed in a high percentage of breast tumors (6). Of 41 primary breast tumor samples quantified by Western blotting relative

to cytokeratin 18, BRK was overexpressed five fold or more in 27%, and overexpressed two fold or more in 61%, relative to normal breast tissue. One line expressed 43-fold higher levels of BRK protein (6).

The Nuclear RNA-binding protein Sam68 is a substrate of BRK

Sam68 (Src associated in mitosis, 68 kDa) is an RNA binding protein (8) that was first identified as a major target of Src during mitosis (9, 10). Sam68 has been shown to preferentially bind RNA with UAAA motifs (11), and has been observed to localize in novel nuclear bodies called Sam68/SLM nuclear bodies (SNBs) in cancer cell lines (12). Although the function of Sam68 is unknown, Sam68 has been shown to be required for cell cycle progression (13), and can function as a cellular homolog of Rev by transporting unspliced HIV mRNA into the cytoplasm (14).

Sam68 coimmunoprecipitated with BRK from nuclear extracts of human MCF-7 and HT29 breast and colon carcinoma cells (15). BRK colocalized with Sam68 within SNBs within the nuclei of these cells. In co-transfection studies Sik phosphorylated Sam68 within the nucleus. Phosphorylation of Sam68 by Sik negatively regulated its RNA binding ability and its ability to function as a Rev cellular homologue (15). Phosphorylation of Sam68 by BRK within the nucleus may have important physiological significance and may contribute to the posttranscriptional control of gene expression during the differentiation of epithelial linings.

BODY

We hypothesize that BRK contributes breast cancer by phosphorylating specific protein substrates such as Sam68, leading to changes in gene expression at the protein level. The goal of the funded concept grant was to identify proteins that are differentially expressed due to changes in BRK activity, as well as additional substrates of this tyrosine kinase to better understand its role in the etiology of breast cancer.

Work proposed in the Concept grant *(taken from submitted proposal)*

Wildtype and super-activated BRK will be introduced into the normal murine mammary gland cell line NMuMG that does not express BRK. Phosphorylated proteins and new protein expression will be identified by comparing protein phosphorylation and protein expression between the parental NMuMG cell line lacking BRK and NMuMG cells expressing activated BRK. In parallel, an inhibitory dominant negative form of BRK in which the catalytic domain has been mutated will be introduced into breast tumor cells that express high levels of BRK. Expression of the dominant negative protein will

eliminate BRK activity in these cells, and we may see a reduction in tyrosine phosphorylation and alterations in the expression of proteins regulated by BRK activity. Differentially phosphorylated and expressed proteins in these modified normal and tumor cell lines will be examined using two-dimensional (2-D) gel electrophoresis.

Since we will compare protein expression patterns in cells that differ only in expression of BRK, we do not expect complex changes in protein expression. In addition to seeing differentially expressed proteins, migration of proteins that are substrates may be altered due to changes in tyrosine phosphorylation. Immunoblotting will be performed with anti-phosphotyrosine antibodies to identify BRK substrates. If we identify unique protein spots that appear or disappear as a result of altering BRK expression, or unknown proteins that are differentially phosphorylated, preparative gels will be run and the unique protein spots will be excised and sent a facility with expertise in mass spectroscopy and bioinformatics.

Key Research Accomplishments

Brk expression constructs have been introduced into the NMuMG cell line and the MCF7 cell lines and stable cell lines have been selected, isolated, and characterized. Protein extracts and mRNA preparations have been made, and we are in the process of comparing patterns of protein and mRNA expression between the cell lines. Currently we are still working out the needed methodology for the proteomics experiments. One set of microarray experiments was performed and the data obtained are currently being analyzed.

During the course of our studies we identified an additional tyrosine kinase that may have overlapping functions with BRK. Genomic sequence became available (AL121907.20, AL121829.29) that links human Brk within four kilobases of a diverged Src-like kinase with similar structure, Srm **(Figure 1)**. Close linkage of the BRK and Srm genes was confirmed by mapping of the murine orthologues of each to distal chromosome 2 (5, 16). The human Srm has not been cloned as a cDNA, but if nonhomologous EST transcripts appropriately oriented and polyadenylated extending to 2329 bp following the conserved stop codon are assumed to represent Srm transcripts, the distance between the presumed polyadenylation site of Srm (AA534748.1, AI762098.1) and the initiation site of Brk (17) is only 1112 bp. Our analysis of the genomic sequence also establishes that Srm and Brk share a precisely conserved exon structure, with seven introns at identical positions in each transcript.

While BRK has a low degree of homology to other intracellular tyrosine kinases including Srm, Frk/Rak, and Src42A/Dsrc41, the exon structures of these kinases is

highly conserved **(Figure 2)**, and distinct from other families of intracellular kinases including c-Src. Frk/Rak, is also expressed at high levels in breast cancer, suggesting a general role for the BRK family of kinases in breast cancer (18, 19).

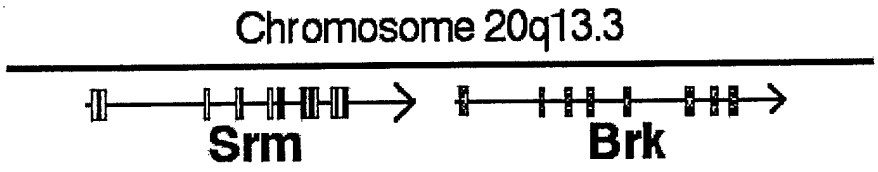

Figure 1. Tight linkage of *Brk* and *Srm* on chromosome 20. Genes mapping to human chromosome 20q13.3 are diagrammed. The murine genes are also tightly lined and map to distal chromosome 2 (5, 16).

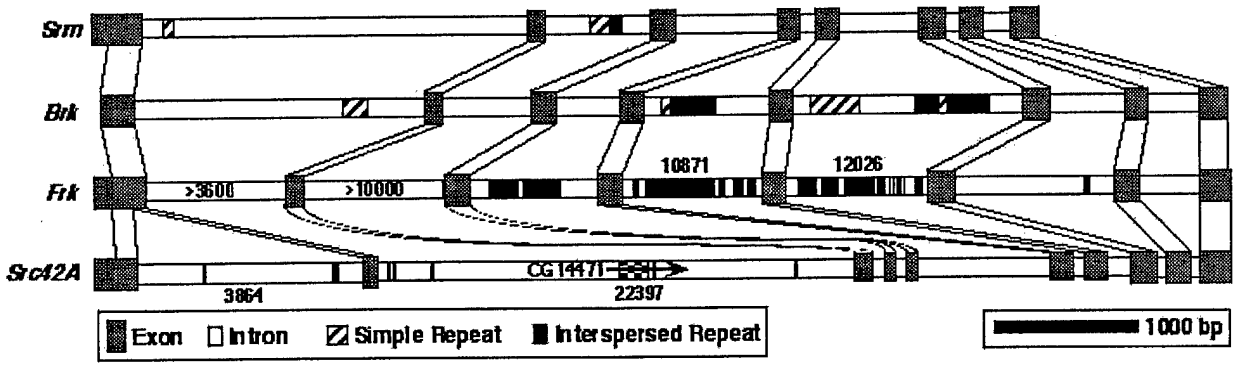

Figure 2. Comparison of the exon structures of Brk family members. Homologous exons 1 to 8, excluding 5' and 3' UTR, comprising the coding sequence of each protein, are depicted for each, with introns drawn to scale except as indicated for Frk introns. Simple repeats (excluding low complexity sequence) and interspersed repeats predicted by RepeatMasker are shown (Smit, AFA & Green, P. RepeatMasker at http://ftp.genome.washington.edu/RM/RepeatMasker.html). Introns with marked sizes are not drawn to scale. Note that much of the intron length variation can be accounted for by recent insertions of primate-specific interspersed repeats, which have targeted the less G+C-rich sequence of Frk far more heavily. The gene CG14471 is a hypothetical Drosophila protein located in an intron of Src42A.

Srm was originally cloned by PCR amplification from neural precursor cells of 10-day mouse embryos, and is expressed in adult lung, testes, and liver at highest levels, in spleen, kidney, and ovary at moderate levels, and at low levels in intestine, muscle, thymus, heart, cerebellum, and cerebrum (16). No information was available about its

expression in the mammary gland. We used PCR to amplify a portion of the murine Srm cDNA and we have generated a probe for performing RNase protection experiments. Experiments are currently underway to examine the expression of this kinase in breast development and in human breast cancer.

Reportable Outcomes

A manuscript describing the tight linkage of BRK/Sik and the related kinase Srm and possible evolutionary consequences is in preparation.

Michael S. Serfas and Angela L. Tyner. Brk, Srm, Frk, and Src42A form a distinct family of intracellular Src-like tyrosine kinases. In preparation.

Conclusions

Our ongoing experiments will lead to the identification of proteins that are differentially expressed due to induction of BRK and new substrates of BRK, and will lead to a better understanding of signal transduction pathways regulated by BRK in breast cancer. These proteins may provide new prognostic indicators for breast cancer and be potential targets for therapeutic intervention.

Our analysis of available genome sequence data has rasied questions about possible co-regulation and expression of the tightly linked Srm tyrosine kinase gene in breast cancers. Expression of this related protein is currently under investigation.

References

1. Mitchell, P.J., K.T. Barker, J. Shipley, and M.R. Crompton, Characterisation and chromosome mapping of the human non receptor tyrosine kinase gene, brk. Oncogene, 15: 1497-1502; 1997.
2. Lee, H., M. Kim, K.H. Lee, K.N. Kang, and S.T. Lee, Exon-intron structure of the human PTK6 gene demonstrates that PTK6 constitutes a distinct family of non-receptor tyrosine kinase. Mol Cells, 8: 401-407; 1998.
3. Lee, S.T., K.M. Strunk, and R.A. Spritz, A survey of protein tyrosine kinase mRNAs expressed in normal human melanocytes. Oncogene, 8: 3403-3410; 1993.
4. Mitchell, P.J., K.T. Barker, J.E. Martindale, T. Kamalati, P.N. Lowe, M.J. Page, B.A. Gusterson, and M.R. Crompton, Cloning and characterisation of cDNAs encoding a novel non-receptor tyrosine kinase, brk, expressed in human breast tumours. Oncogene, 9: 2383-2390; 1994.

5. Llor, X., M.S. Serfas, W. Bie, V. Vasioukhin, M. Polonskaia, J. Derry, C.M. Abbott, and A.L. Tyner, BRK/Sik expression in the gastrointestinal tract and in colon tumors. Clin Cancer Res, 5: 1767-1777; 1999.
6. Barker, K.T., L.E. Jackson, and M.R. Crompton, BRK tyrosine kinase expression in a high proportion of human breast carcinomas. Oncogene, 15: 799-805; 1997.
7. Easty, D.J., P.J. Mitchell, K. Patel, V.A. Florenes, R.A. Spritz, and D.C. Bennett, Loss of expression of receptor tyrosine kinase family genes PTK7 and SEK in metastatic melanoma. Int J Cancer, 71: 1061-1065; 1997.
8. Wong, G., O. Muller, R. Clark, L. Conroy, M.F. Moran, P. Polakis, and F. McCormick, Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. Cell, 69: 551-558; 1992.
9. Taylor, S.J. and D. Shalloway, An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis. Nature, 368: 867-871; 1994.
10. Fumagalli, S., N.F. Totty, J.J. Hsuan, and S.A. Courtneidge, A target for Src in mitosis. Nature, 368: 871-874; 1994.
11. Lin, Q., S.J. Taylor, and D. Shalloway, Specificity and determinants of Sam68 RNA binding. Implications for the biological function of K homology domains. J Biol Chem, 272: 27274-27280; 1997.
12. Chen, T., F.M. Boisvert, D.P. Bazett-Jones, and S. Richard, A Role for the GSG Domain in Localizing Sam68 to Novel Nuclear Structures in Cancer Cell Lines. Mol Biol Cell, 10: 3015-3033; 1999.
13. Barlat, I., F. Maurier, M. Duchesne, E. Guitard, B. Tocque, and F. Schweighoffer, A role for Sam68 in cell cycle progression antagonized by a spliced variant within the KH domain. J Biol Chem, 272: 3129-3132; 1997.
14. Reddy, T.R., W. Xu, J.K. Mau, C.D. Goodwin, M. Suhasini, H. Tang, K. Frimpong, D.W. Rose, and F. Wong-Staal, Inhibition of HIV replication by dominant negative mutants of Sam68, a functional homolog of HIV-1 Rev. Nat Med, 5: 635-642; 1999.
15. Derry, J.J., S. Richard, H. Valderrama Carvajal, X. Ye, V. Vasioukhin, A.W. Cochrane, T. Chen, and A.L. Tyner, Sik (BRK) Phosphorylates Sam68 in the Nucleus and Negatively Regulates Its RNA Binding Ability. Mol Cell Biol, 20: 6114-6126; 2000.
16. Kohmura, N., T. Yagi, Y. Tomooka, M. Oyanagi, R. Kominami, N. Takeda, J. Chiba, Y. Ikawa, and S. Aizawa, A novel nonreceptor tyrosine kinase, Srm: cloning and targeted disruption. Mol Cell Biol, 14: 6915-6925; 1994.

17. Vasioukhin, V., M.S. Serfas, E.Y. Siyanova, M. Polonskaia, V.J. Costigan, B. Liu, A. Thomason, and A.L. Tyner, A novel intracellular epithelial cell tyrosine kinase is expressed in the skin and gastrointestinal tract. Oncogene, 10: 349-357; 1995.
18. Meric, F., W.P. Lee, A. Sahin, H. Zhang, H.J. Kung, and M.C. Hung, Expression profile of tyrosine kinases in breast cancer. Clin Cancer Res, 8: 361-367.; 2002.
19. Cance, W.G., R.J. Craven, M. Bergman, L. Xu, K. Aitalo, and E.T. Liu, Rak, a novel nuclear tyrosine kinase expressed in epithelial cells. Cell Growth Diff., 5: 1347-1355; 1994.

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